Kidney International, Vol. 29 (1986), pp. 971–976

Characterization of monoclonal antibodies specific for human Tamm–Horsfall protein

M. Brunisholz, M. Geniteau-Legendre, Pierre M. Ronco, Phillippe Moullier, F. Pontillon, Gabriel Richet, and Pierre J. Verroust

Unité Inserm U. 64, Hôpital Tenon, 4 rue de la Chine, 75970 Paris Cedex 20, France

Production of monoclonal antibodies specific for human Tamm–Horsfall protein. Fifteen monoclonal antibodies have been produced to human Tamm–Horsfall protein (THP), identifying at least seven distinct epitopes. The antibodies have been used to isolate from serum an immunoreactive protein which comigrates with urinary THP. In addition, the antibodies may prove useful to set up an immunoenzymoassay for urinary THP as well as for immunoaffinity purification.

Tamm–Horsfall protein (THP) is the most abundant protein in normal urine, and the constituent of all tubular casts [1–3]. Its synthesis and membrane expression by cells of the thick ascending limb of the loop of Henle have led to the suggestion that it might be involved in ion transfer [3, 4]. Recently Hoyer et al [5–7] reported experimental studies demonstrating the pathogenic role of active or passive anti-THP immunization. However, the precise functions of THP and its role in human pathology, although likely, are yet undefined. Further studies, including analysis of extra–renal distribution and measurement in body fluids (8–12), remain difficult partly because of the lack of reliable immunological reagents. This led us to undertake the production of monoclonal antibodies (Mab) specific for human THP. We report here on the characterization of the specificities obtained and their use to develop an immunoenzymoassay (IEA) and to demonstrate the presence of THP in serum.

Methods

Preparation of human Tamm–Horsfall protein

Tamm–Horsfall protein was prepared from normal human urine by precipitation with 0.58 M NaCl as previously described [2]. Purity was assessed by polyacrylamide gel electrophoresis in the presence of SDS (PAGE–SDS) [13].

Production of monoclonal antibodies

Monoclonal antibodies were prepared according to methods previously described [14, 15]. Supernatants were screened for the presence of anti-THP antibodies by indirect immunofluorescence (IIF) on frozen kidney sections and by IEA using THP (200 ng in 200 μliter of carbonate bicarbonate buffer, pH 9.4) adsorbed to plastic wells of microtiter plates (Nunc) by incubation for three hr at 37°C. After washing with PBS Tween 0.05% (PBST), the wells were incubated for two hr at room temperature with 100 μliter of supernatant diluted one-half in PBST, washed and incubated with peroxidase labeled sheep anti-mouse IgG [15]. Enzyme activity was revealed with Orthophenylenediamine (OPD). All supernatants considered as positive reacted in the IEA and gave by IIF the typical pattern previously described [3]. Cell cloning, class and subclass determination of the Mab, purification and labeling with biotin were performed as previously described [14–16].

Specificity analysis of the Mab. In order to individualize the epitopes identified by the various Mab, binding of a given biotin labelled Mab (B-Mab) to solid phase THP was assessed in the absence or in the presence of the Mab to be tested. Plastic wells on which THP had been adsorbed as described above were incubated with 100 μliter of supernatant or serial dilutions (10^{-2} to 10^{-7}) in PBST of the ascites containing the Mab analyzed, immediately followed by B-Mab (0.1 μg/mliter) in PBST. Binding was compared to that observed with B-Mab diluted in PBST only. Control experiments were performed with myeloma proteins devoid of known antibody specificity. In all cases, solid phase associated B-Mab was detected and quantified using peroxidase labelled avidin (Vector Laboratories, Compiègne, France) diluted 1/3000 in 50 mM phosphate buffer pH 8.0 containing 0.5 M NaCl and 0.5% Tween 20.

The ability to bind radiolabelled human THP was tested for all Mab. THP was labelled with ^{125}I by the chloramine T method as described by Hartmann et al [17] to a specific activity of 70 μci/μg. A constant amount of antibody was incubated with increasing amounts of radiolabelled ^{125}I THP. After incubation at room temperature for 30 min, Mab as well as antibody–bound THP were removed from the solution by sequential addition of rabbit anti-mouse IgG (5 μg) followed by 50 μliter of a 10% suspension of protein A containing Staphylococci. After washing of the bacterial pellet, the amounts of bound and free THP were measured. Association constants were determined according to Scatchard analysis.

In addition to their fine specificity for human THP, individual Mab were analyzed by IIF on frozen kidney sections for reactivity with rabbit or rat THP.

Received for publication March 12, 1985,
and in revised form August 6, 1985

Use of monoclonal antibodies to develop an IEA for THP

Using the specificity analysis described above, it was possible to develop a capture assay for THP in which a Mab of known specificity was adsorbed to plastic wells and used to insolubilize the THP present in the sample. The amount of THP associated with the solid phase was assessed using a B-Mab specific for a different epitope. The experimental conditions used involved: 1) adsorption of the Mab (200 μliter of a 1 μg/mliter solution in carbonate–bicarbonate buffer) on 96 well microtiter plates for three hours at 37°C; 2) incubation of the sample (200 μliter) for two hr at room temperature in PBST containing 0.005% SDS; 3) incubation with B-Mab at a concentration of 0.1 μg/mliter in PBST for three hr at room temperature. The various steps were separated by extensive washing in PBST. B-Mab was detected using peroxidase labelled avidin as described above. In routine experiments, mixtures of two antibodies were used in steps 1 and 3. Control experiments included the following: omission of the first Mab adsorption step or adsorption on the wells of MOPC 173, a monoclonal IgG devoid of anti-THP antibody activity; incubation with solutions which did not contain THP; and incubation with B-Mab devoid of anti-THP antibody activity.

Detection of THP in normal human serum

The assay was used to demonstrate the presence of immunoreactive THP in fresh human serum. In initial experiments, tenfold serial dilutions of normal human serum were introduced in the capture IEA described above. In order to confirm the specificity of the binding observed, 10 mliter of human serum were passed over a 0.5 mliter column of Sepharose 4B (Pharmacia, Velizy, France) coupled to a monoclonal anti-THP antibody (Mab174) at a concentration of 10 mg/mliter according to manufacturer's instructions. After thorough washing, the bound material was eluted with either alkaline buffer [18], or SDS containing sample buffer [13] and dialysed against distilled water. The reactive material diluted in PBST containing 0.005% SDS was assayed using various combinations of capture and detection antibodies to determine whether the different epitopes defined on urinary THP were detectable on the immunoreactive material. In some experiments, in order to assess the molecular wt of the reactive protein, the SDS–eluted material was separated by PAGE–SDS [13] and transferred to nitrocellulose sheets [19]. The latter were revealed with B-Mab anti-THP followed by peroxidase labelled avidin. Peroxidase reaction product was detected using 4-chloronaphtol. Control experiments were performed using Sepharose 4B coupled to MOPC 173, a monoclonal mouse IgG devoid of known antibody specificity. The eluates were analyzed by IEA and immunoblotting in the same fashion as described above. In addition, whole human serum and urine samples were submitted to PAGE–SDS and analyzed by immunoblotting.

Fractionation of THP from normal urine by affinity chromatography. Two hundred mliter of fresh normal urine collected in the presence of sodium azide were incubated overnight at 4°C with 5 mliter of S4B coupled to a Mab anti-THP. After thorough washing, the bound material was eluted as above, concentrated by lyophilisation, and analyzed by PAGE–SDS.

Table 1. Analysis of the epitopes identified by Mab Anti-TH

B-Mab*	18	33	35	89	104	174	214	195
Inhibitor								
18	+							
109	+							
110	+							
111	+							
202	+							
3						+		
146						+		
174						+		
104					+			
212					+			
33		+						
35			+					
89				+				
195								+
214							+	

Symbols are: *B-Mab, biotine labelled monoclonal antibody; and + indicates complete inhibition when B-Mab is incubated with an excess of unlabelled Mab.

Results

Production of monoclonal anti-THP antibodies

Two hundred and forty of the 300 seeded wells contained hybrids, 43 of which secreted antibody reactive with renal tissue. Fifteen reacted with THP by immunoenzymoassay and indirect immunofluorescence on frozen kidney sections. The 28 remaining antibodies did not react with THP but were able to bind various renal structures including proximal tubule brush border.

Specificity of monoclonal anti-THP antibodies

In order to analyze the fine specificity of the anti-THP antibodies, the first two antibodies which were cloned, Mab 18 and Mab 174, were purified and labelled with biotin. No cross inhibition was found when the binding of each of these B-Mab to solid phase THP was studied in the presence of the other Mab, thus indicating that the two Mab were directed against distinct epitopes. At this stage, supernatants from each of the hybrids obtained were tested for their ability to inhibit binding of the two B-Mab. Supernatants which did not inhibit binding in any of the two systems were considered as defining a new epitope. The corresponding cells were cloned, and the Mab so produced was purified, labelled with biotin and added to the original panel. The selection process was then repeated with the new extended panel. Table 1 summarizes the results of cross–inhibition experiments. Antibodies classified as directed against the same epitope were equally efficient to inhibit binding of the reference B-Mab. The results initially obtained with the supernatants were confirmed on dilutions of the corresponding ascites, as illustrated in Figure 1. In most instances, as shown in panel A, when Mab against distinct epitopes were compared, inhibition was only obtained when the homologous Mab was added to the B-Mab. Panel B illustrates the exception to this rule: Mab 174 was able to inhibit partially the binding of B-Mab 35, whereas Mab 35 was unable to produce any inhibition of binding of B-Mab 174. Thus complete inhibition was only obtained when the homologous Mab was added to the B-Mab.

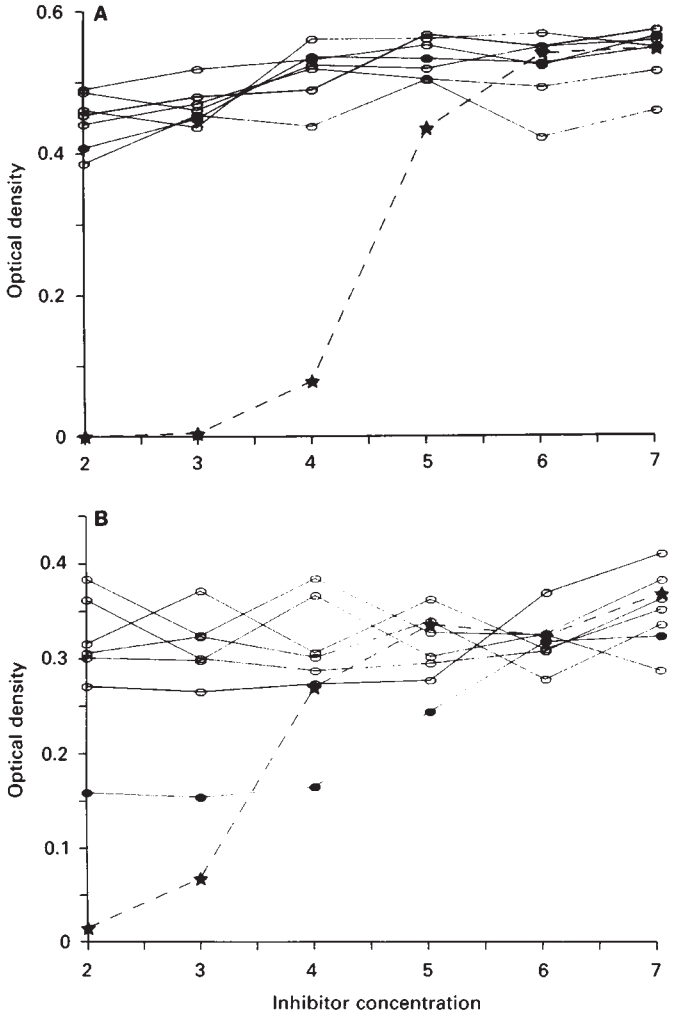

Fig. 1. *Analysis of the specificity of eight monoclonal anti-THP antibodies, Mab 18, 33, 35, 89, 104, 174, 214, 195, directed against distinct epitopes.* **1A.** Binding of biotin labelled Mab 174 was studied in the absence of competitor or in the presence of the various unlabelled Mab (ascites diluted 10^{-2} to 10^{7}). Inhibition is only observed in the presence of unlabelled Mab 174 (★). Note lack of inhibition by any of the other Mab (○) including Mab 35 (●). **1B.** Binding of biotin labelled Mab 35 was studied under similar conditions. Complete inhibition is only observed in the presence of unlabelled Mab 35 (★), but partial inhibition is detectable with Mab 174 (●). Inhibition was not detected with any of the other Mab (○).

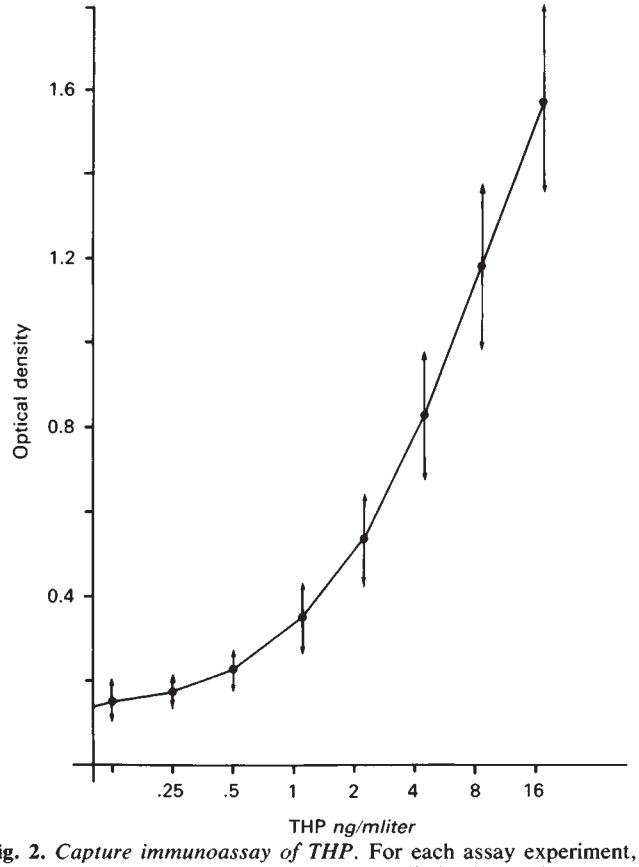

Fig. 2. *Capture immunoassay of THP.* For each assay experiment, a standardization curve was constructed by offering increasing amounts of THP (abscissa) and measuring the optical density obtained (ordinate). Each point is the mean ± SD of all tests performed over a period of three months.

Whereas all the Mab obtained were able to bind unlabelled THP, only Mab 174, 3 and 146 could bind ^{125}I labelled THP. The binding data was analyzed according to Scatchard. The KA was found to be $2 \times 10^{7} M^{-1}$.

Most epitopes identified by the Mab were specific for human THP. Mab 214 however reacted with rabbit and rat THP. Mab 195 displayed reactivity for rat THP only, Mab 89 and 174 for rabbit THP only.

Development of an immunoenzymoassay for THP

The specificity analysis was used to set up a capture IEA. Mab 89 and 104 were first adsorbed to plastic wells which were subsequently incubated with THP containing solutions. After washing the wells were incubated with B-Mab 174 and B-Mab 18. The assay was very reproducible, as shown in Figure 2 which depicts means ± SD of all optical densities measured when serial twofold dilutions of THP were used to construct standard curves over a period of three months. Specificity of the assay was demonstrated by the lack of binding when the anti-THP antibodies adsorbed to the wells were omitted (MOPC 173 coated wells). Sensitivity of the assay allowed detection of THP concentrations as low as 1 ng/mliter. In order to compare reactivity of THP under various conditions, binding curves were constructed using serial two fold dilutions of reference purified THP and unfractionated urine. The slopes of the linear segments ($r < 0.995$) were not statistically different. Intra- and interassay coefficients of variation are respectively 10% and 13%.

Detection of THP in normal serum

As illustrated in Figure 3, the IEA was used to detect the presence of THP in serum. Levels measured varied from 5 to 50 ng/mliter. Using various combinations of capture and detection Mab, it was possible to demonstrate (Fig. 4) that all the five epitopes tested, initially identified on urinary THP, were present on the immunoreactive material prepared from serum. The IEA was used to construct binding curves with serial twofold dilutions of the reference THP and of the material eluted from S4B-Mab 174. The slopes of the linear segments of the two

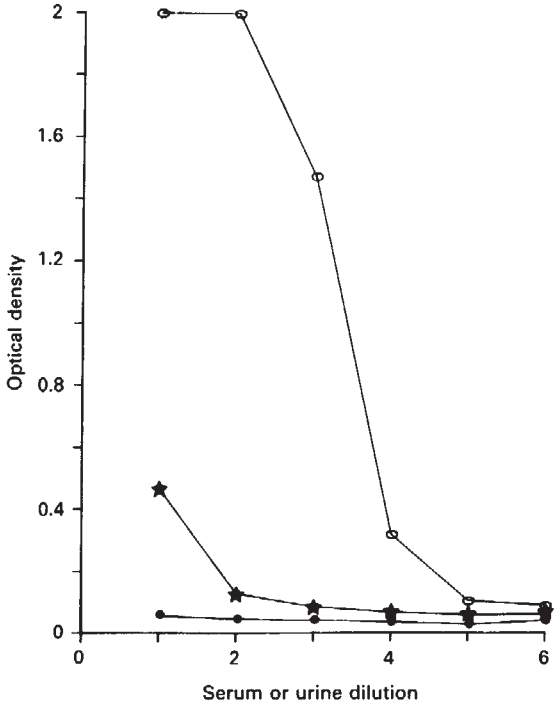

Fig. 3. *Detection of immunoreactive material in normal human serum.* Serial dilutions (10^{-1} to 10^{-6}) of normal serum or normal urine were introduced in the assay system described in text, and optical densities were measured. Reactivity was observed with normal serum (★) and urine (○). Note lack of binding when capture antibodies are omitted (●).

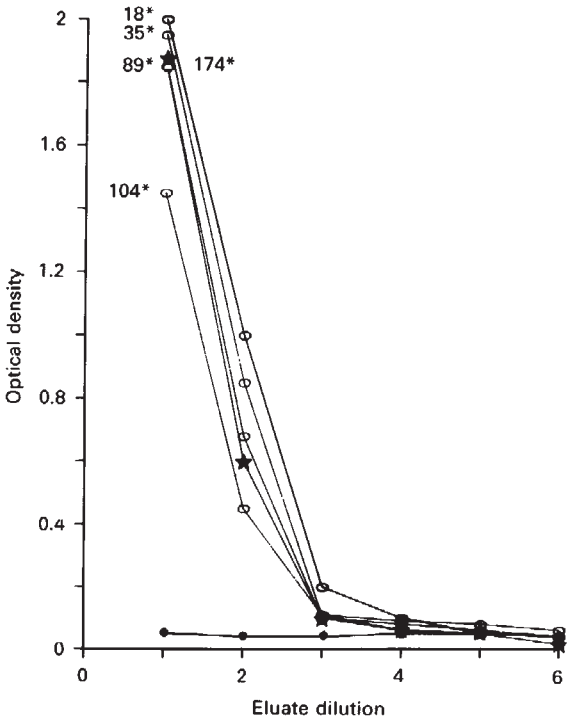

Fig. 4. *Characterization of immunoreactive material detected in normal serum.* In a first series of experiments, assay wells were first coated with Mab 89 and Mab 174 (○) and used to capture immunoreactive material prepared by affinity chromatography from normal serum. Bound material was detected with one of the following B-Mab (*) 18, 35, 89 or 104. In a second series of tests, wells were coated with Mab 89 and Mab 18 (★). Bound material was detected with B-Mab 174. Reactive material prepared from serum could be detected using all antibody combinations. Note lack of reactivity of wells coated with MOPC 173 (●).

curves were not statistically different. Recovery experiments were performed in which increasing amounts of THP were added to normal human serum. The values for measured THP were plotted against the expected values. The regression line obtained has an r value of 0.996 and a slope of 1.06, thus indicating satisfactory recovery. Immunoreactive material could not be detected in control experiments using S4B-MOPC 173.

In order to assess the molecular wt of the immunoreactive material present in serum, material eluted from the immunoadsorbent with SDS buffer was submitted to PAGE–SDS, transferred to nitrocellulose sheets and revealed using B-Mab. Preliminary experiments demonstrated that THP prepared from normal urine and analyzed in the same manner was reactive with the various Mab; Mab 174 and 18 were the best reactors and Mab 89 the poorest. As shown in Figure 5, it was possible using B-Mab 174 to identify clearly a major immunoreactive band comigrating with THP prepared from normal urine. No specific binding was found when normal human serum, or eluates from S4B-MOPC 173, were separated by PAGE–SDS and analyzed by immunoblotting with the various B-Mab.

Preparation of urinary THP by affinity chromatography. As shown in Figure 6, when the material eluted from Mab 174 or Mab 18 bound to Sepharose 4B was analyzed by PAGE–SDS, a single band comigrating with THP prepared by salt precipitation was obtained.

Discussion

The results presented in this paper show that a variety of Mab anti-human THP can be obtained. It is interesting to note that a number of Mab were produced to specificities unrelated to THP. This may be in relation with the ability of THP to bind unrelated proteins which may thus be present in the purified preparations. The specificity studies clearly indicate that the Mab individualize seven distinct epitopes. It cannot be ascertained whether Mab 174 and Mab 35 bind closely related epitopes with consequent steric hindrance, or whether they recognize the same epitope but with greater affinity for Mab 174. The latter hypothesis is difficult to reconcile with the fact that inhibition reaches a plateau at 50% when unlabelled inhibitor is increased over two orders of magnitude. The specificity of the Mab provides several informations on the immunological reactivity of THP. First, most of the Mab were unable to react with iodinated THP, suggesting that THP may be very sensitive to oxidative damage by chloramine T and/or introduction of iodine in the tyrosine residues. Second, some epitopes such as that defined by Mab 18 may be more immunogenic than others as indicated by the isolation of five reactive clones. Third, only a minority of Mab were able to react with antigenic determinants present in several species, suggesting that they might be less immunogenic.

The antibodies obtained were adequate to set up a capture IEA for THP. Such an assay had not been previously reported and was found to be as sensitive as the radioimmunoassays at present available [8–11]. The reproducibility of the IEA and the

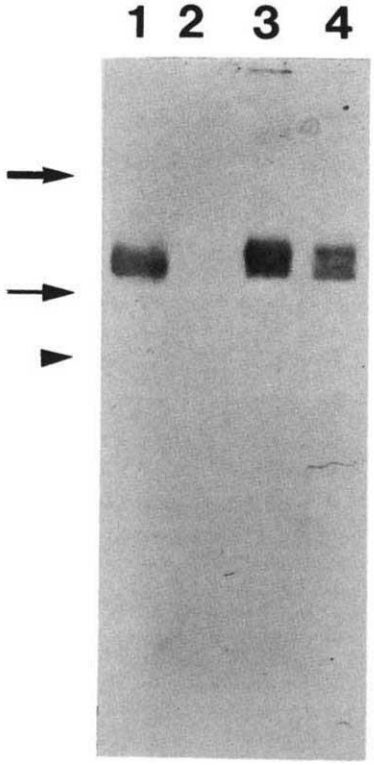

Fig. 5. *Analysis of immunoreactive material present in serum.* THP was purified from urine (lane 1), and serum in two different experiments (lanes 3 and 4) by immunoaffinity chromatography on Sepharose 4B coupled to Mab 174. Lane 2 is a control eluate prepared from normal human serum using Sepharose 4B coupled to MOPC 173 (a control monoclonal mouse IgG devoid of anti-THP activity) and analyzed in the same manner. Molecular wt markers include unreduced IgG (→), μ chain of IgM (→) and γ chain of IgG (▶).

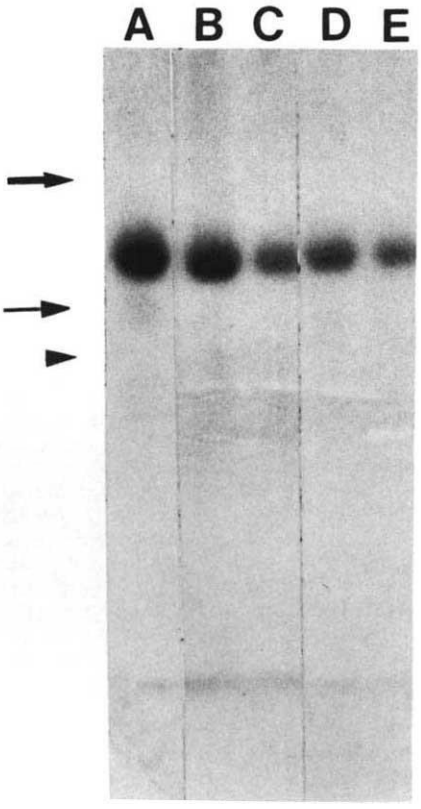

Fig. 6. *Preparation of THP from normal urine by immunoaffinity chromatography.* PAGE–SDS stained with Coomassie Blue. A, THP prepared by salt precipitation; B–E, THP prepared by immunoaffinity chromatography in four different experiments. Note identical patterns. Symbols are as in **Fig. 5.**

indefinite availability of the reagents should be of assistance to standardize THP measurements already made difficult by the variability of THP reactivity [20]. The sensitivity of the assay was sufficient to study THP reactive material in normal human serum. Studies using polyclonal antisera suggested that THP was detectable in normal sera, but cross reactivity could not be excluded. In fact, this possibility was supported by PAGE–SDS analysis of the material purified by affinity chromatography which demonstrated [21] the presence of numerous proteins, none of which comigrating with purified THP. Using developments of the EIA described in this work, it could be shown that the reactive material detectable in normal human serum shares at least five different epitopes with urinary THP. Binding curves obtained with serum and urine-derived material were parallel suggesting identical reactivity. In addition, immunoblotting experiments established that the reactive material isolated from serum migrated on polyacrylamide gels with the expected molecular wt for THP, thus practically excluding cross reactivity. The development of a simple and reproducible IEA should allow further clinical studies of THP in renal pathology such as pyelonephritis [22], tubulointerstitial diseases [24–26], tubular obstruction [24] or transplant rejection [27].

The potential applications of the Mab reported are not limited to assays. Using immunoadsorption techniques, it has been possible to prepare THP by methods which do not involve high salt precipitation, and can be performed in a few hrs. Because the relations between structure and function are probably of primary importance, the specificity of the Mab may be of considerable value for further studies.

In conclusion, 15 Mab specific for THP have been produced. They define at least seven different epitopes and may be used for IEA and immunoaffinity purification.

Reprint requests to Dr. Pierre Verroust, Unité Inserm U. 64, Hôpital Tenon, 4 rue de la Chine, 75970 Paris Cedex 20, France

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